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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

Docket Number 240574US0PROV

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TITLE OF THE INVENTION (280 CHARACTERS MAX)

PKNB KINASE ACTIVITY IS REGULATED BY PHOSPHORYLATION IN TWO THR RESIDUES AND DEPHOSPHORYLATION BY PSTP, THE COGNATE PHOSPHO-SER/THR PHOSPHATASE, IN MYCOBACTERIUM TUBERCULOSIS

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PROVISIONAL APPLICATION COVER SHEET

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PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*

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Summary

Bacterial genomics revealed the widespread presence of eukaryotic-like protein kinases and phosphatases in prokaryotes, but little is known on their biochemical properties, regulation mechanisms and physiological roles. Here we focus on the catalytic domains of two *trans*-membrane enzymes, the Ser/Thr protein kinase PknB and the protein phosphatase PstP from *Mycobacterium tuberculosis*. PstP was found to specifically dephosphorylate model phospho-Ser/Thr substrates in a Mn^{2+}-dependent manner. Autophosphorylated PknB was shown to be a substrate for Pstp and its kinase activity was affected by PstP-mediated dephosphorylation. Two threonine residues in the PknB activation loop, found to be mostly disordered in the crystal structure of this kinase, namely Thr171 and Thr173, were identified as the target for PknB autophosphorylation and PstP dephosphorylation. Replacement of these threonine residues by alanine significantly decreased the kinase activity, confirming their direct regulatory role. These results indicate that, as for eukaryotic homologues, phosphorylation of the activation loop provides a regulation mechanism of mycobacterial kinases and strongly suggest that PknB and PstP could work as a functional pair *in vivo* to control mycobacterial cell growth.

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Introduction

Tuberculosis (TB) is a major public health problem with one-third of the world's population infected by its aetiologic agent, *Mycobacterium tuberculosis*, and over two million people dying from the disease each year (Dye *et al.*, 1999) (http://www.who.int). The Global Alliance for TB Drug Development has proposed that the current treatment could be improved considerably by developing more potent therapeutic agents, that reduce the duration of therapy, and by including drugs that act on latent bacilli (Global Alliance for TB Drug Development, 2001). Faced with the urgency to develop new therapeutic strategies, it appears crucial to understand better the physiopathology of the causative agent and its complex relationship with the immune system of the host. [1]

After inhalation, infectious bacilli are phagocytosed by alveolar macrophages in the lung and induce a local pro-inflammatory response, which leads to the recruitment of monocytes from the bloodstream into the site of infection (Dannenberg, 1999; Russell, 2001). By blocking fusion of phagosomes with lysosomes in these non-activated macrophages (Brown *et al.*, 1969; Sturgill-Koszycki *et al.*, 1996), *M. tuberculosis* escapes killing and multiplies. As the immune response progresses, macrophages and T cells accumulate to form a granuloma in which the pathogen is contained in a latent state (Parrish *et al.*, 1998; Manabe and Bishai, 2000). It can lie dormant for years only to rise again when the immune system wanes through old age, malnutrition or AIDS (acquired immuno-deficiency syndrome). The centre of the granuloma then liquefies and *M. tuberculosis* replicates profusely and is discharged into the bronchial tree producing an infectious cough (Dannenberg, 1999). To understand the bacterial response to these changes in host environment, the study of regulatory proteins

involved in mycobacterial signal transduction is therefore of the utmost importance.

Phosphorylation, a simple and efficient means of reversibly changing the biochemical properties of a protein, is a major mechanism for signal transduction and regulation of almost all biological functions. There are two main phosphorylative signal transduction systems. Prokaryotes predominantly use the two-component system, comprising in its simplest form a signal sensor with a histidine kinase domain and a response regulator, often a transcriptional factor (Wurgler-Murphy and Saito, 1997; Stock *et al.*, 2000). This simple, unidirectional mechanism allows a quick response to abrupt environmental changes. The second system depends on the reversible phosphorylation of serine, threonine and tyrosine residues, and is widely used in eukaryotes (Hanks and Hunter, 1995; Hunter, 1995; Barford *et al.*, 1998; Hunter, 2000). This mechanism involves the action of protein kinases and phosphoprotein phosphatases in cascades and networks (Hunter, 2000), providing an efficient means for the rapid modulation of the transduced signal to serve highly regulated functions.

Since the identification of the first bacterial homologue a few years ago (Muñoz-Dorado *et al.*, 1991), genomics has now demonstrated that serine, threonine and tyrosine protein kinases and phosphatases are also widespread in prokaryotes (Zhang, 1996; Kennelly, 2002). The two phosphorylation mechanisms (two-component systems and Ser/Thr/Tyr kinases and phosphatases) in prokaryotes may regulate distinct functions or act together in the same signalling pathway. The presence of Ser/Thr and Tyr kinases and phosphatases in prokaryotes appears to be associated with a complex, multistage developmental cycle and possible roles in regulating growth and development (heterocyst, fruiting-body or spore formation) have been proposed (Zhang, 1996; Shi *et al.*, 1998). The dormant state of *M. tuberculosis*, although poorly understood, may be considered in some regards analogous to sporulation (Demaio *et al.*, 1996) and thus involve these enzymes.

Mycobacterium tuberculosis employs both systems of protein phosphorylation. It has 15 sensor His kinases and 15 response regulators, forming at least 11 functional pairs, together with 11 putative Ser/Thr protein kinases (STPKs), one phospho-Ser/Thr phosphatase (*ppp* renamed here *pstP*) and two Tyr phosphatases (*ptpA*, *ptpB*) (Cole *et al.*, 1998) (http://www.genolist.pasteur.fr/TubercuList). There appears to be no counterpart Tyr kinase for the two Tyr phosphatases, PtpA and PtpB, which can, moreover, be secreted (Koul *et al.*, 2000; Cowley *et al.*, 2002). Eight of the 11 STPKs are predicted to be transmembrane proteins, with a putative extracellular signal sensor domain and an intracellular kinase domain. Six STPKs (PknA, B, D, E, F, G) have already been expressed as recombinant proteins and shown to be functional kinases (Peirs *et al.*, 1997; Avenue-Gay *et al.*, 1999; Koul *et al.*, 2001; Chaba *et al.*, 2002; data not shown for PknE).

At this time, no physiological role has been clearly demonstrated for any of the STPKs or phosphatases from *M. tuberculosis*, and knock-out mutants have not yet been reported. Here, we have focused our interest on PknB and PstP as indirect data suggest they could play an essential role in the biology of *M. tuberculosis*. The *pknB* and *pstP* genes along with *pknA* are found in an operon (Fig. 1) that also includes *rodA* and *pbpA* (Cole *et al.*, 1998), two genes encoding morphogenic proteins involved in peptidoglycan synthesis during cell growth (Matsuhashi, 1994). Furthermore, this genomic region remains unchanged in the close relative *M. leprae* (Fsihi *et al.*, 1996), in spite of the extensive gene decay in this bacillus which has removed or inactivated over 2400 genes including those for all other STPKs (except for PknL and PknG) and both Tyr phosphatases (Eiglmeier *et al.*, 2001). Thus, the conservation of the *pknA*, *pknB* and *pstP* genes near the chromosomal origin of replication in *M. leprae* strongly suggests that the corresponding enzymes could regulate essential functions, possibly related to cell growth or latency of mycobacteria.

We demonstrate here that Pstp dephosphorylates specifically phospho-Ser/Thr residues and that its activity is strictly dependent on the presence of divalent cations. We also report that the catalytic domain of PknB, as defined by homology modelling, is an active protein kinase in its phosphorylated state. Pstp is capable of dephosphorylating PknB, which subsequently exhibits decreased kinase activity. Mass spectrometry analysis identified two phosphothreonine residues in the activation loop of PknB. Mutagenesis of these threonines in alanine demonstrate their role in regulating PknB kinase activity. We suggest that Pstp and PknB could interplay *in vivo* in the same transduction pathway, and discuss the putative regulatory roles of these enzymes in mycobacteria.

Results

PstP is a Ser/Thr protein phosphatase

The *pstP* gene (Rv0018c) encodes a putative transmembrane protein of 514 aa (Cole *et al.*, 1998) with a C-terminal extracellular domain (196 aa) rich in proline and serine residues (Fig. 2A). The putative intracellular domain (301 aa) is homologous to members of the eukaryotic Ser/Thr protein phosphatase PPM family (Bork *et al.*, 1996). The sequence alignment of the catalytic domains of PstP and human PP2C, the prototype member of the PPM family, is shown in Fig. 2B. Although PstP displays only 17% identity with the human enzyme, all the motifs corre-

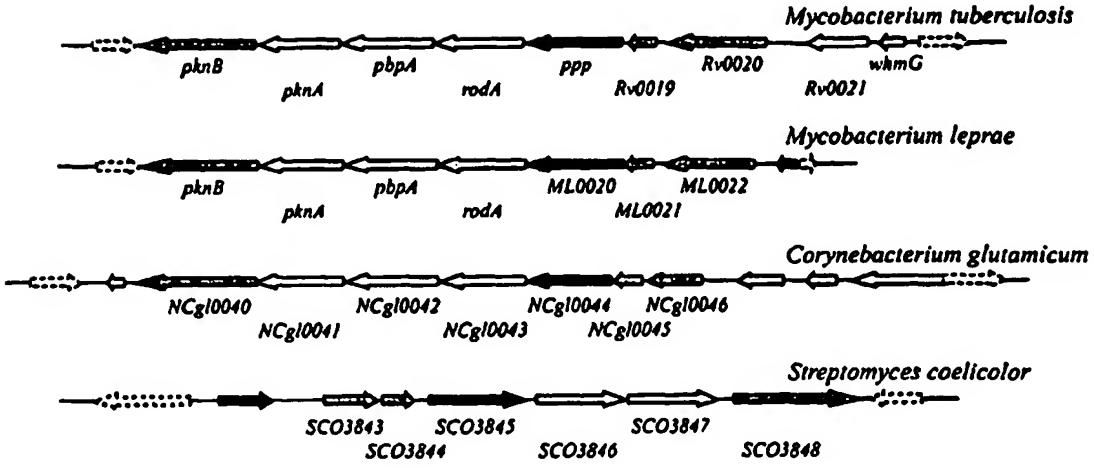

Fig. 1. Conserved structure of the putative operon including the *pknB* and *pstP* genes in several actinobacteria. The genes coding for the following signal tranduction elements, PknA, PknB, PstP and two proteins with a FHA domain, are co-localized with two genes involved in peptidoglycan synthesis, namely *pbpA* and *rodA*. This gene cluster is conserved in all actinobacteridae genomes known to date, including those presented here, *M. tuberculosis* (http://www.genolist.pasteur.fr/TubercuList), *M. leprae* (http://www.genolist.pasteur.fr/Leproma), *C. glutamicum* (http://www.tigr.org) and *S. coelicolor* (http://www.sanger.ac.uk) (note that the *pknA* gene is missing in *S. coelicolor* genome) and also such as *C. diphteriae*, *C. efficiens*, *Thermobifida fusca* and *Bifidobacterium longum* (http://www.ncbi.nlm.nih.gov).

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sponding to key structural elements (Bork *et al.*, 1996) are present in the PstP sequence. The crystal structure of the human PP2C has revealed a metal ion-catalysed dephosphorylation mechanism (Das *et al.*, 1996). As indicated in Fig. 2B, all the residues involved in the binding of metal cations and phosphate are conserved in PstP, suggesting a common mechanism of phosphate recognition and catalysis.

The multiple alignment of PstP with other members of the PPM phosphatase family predicted Asp 240 as the last residue of the catalytic domain. Thus, the His-tagged construction $PstP_{1-240}$ was produced as a soluble protein in *E. coli* (Fig. 3A). The protein phosphatase activity and the specificity towards phospho-amino acids were tested using different substrates. The myelin basic protein (MBP) and α-casein were phosphorylated either on serine and threonine residues with the protein kinase A (PKA) or on tyrosine residues with the Abl kinase using radiolabelled ATP. As shown in Fig. 3B, PstP dephosphorylated phospho-Ser/Thr substrates but showed little or no activity with phospho-Tyr substrates. Furthermore, PstP phosphatase activity was strictly dependent on divalent cations with a preference for Mn^{2+} versus Mg^{2+} (data not shown). Thus, in agreement with sequence homology-based predictions, these results demonstrate that the intracellular region of PstP is a Ser/Thr protein phosphatase that belongs to the PPM family.

The C-terminal domain of PknB is similar to that found in various other bacterial STPKs

PknB is predicted to be a 626 aa transmembrane protein with an intracellular N-terminal kinase domain (331 aa) and an extracellular C-terminal domain (276 aa) (Fig. 4A). This structural organization for STPKs is found in plants and as receptors for the transforming growth factor β (TGFβ) family cytokines in vertebrates, where the C-terminal domain is a signal sensor. This could also be the case for the transmembrane STPKs from prokaryotes. The C-terminal domain of PknB shows some degree of sequence similarity with the C-terminal domain of several prokaryotic STPKs, including actinobacteria (corynebacterium, streptomyces, bifidobacterium) and other Gram-positive bacteria (listeria, bacillus, streptococcus) (Fig. 4B). These proteins display a diverse number of copies, four in PknB, of the recently described PASTA domain (for penicillin-binding-protein and serine/threonine kinase associated domain, Yeats *et al.*, 2002). This suggests that all these kinases could respond to a similar type of ligand. Actually, it has been speculated that the PASTA domains could bind unlinked peptidoglycan (Yeats *et al.*, 2002), although no experimental evidence is available to substantiate this claim. It is noteworthy that a gene coding for a putative Ser/Thr protein phosphatase is found in the same genomic region for the above mentioned organisms,

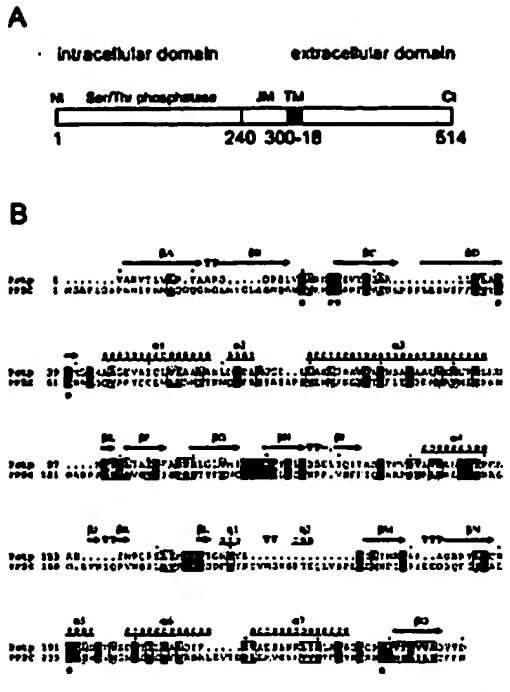

Fig. 2. A. Structural organization of PstP. JM; juxta-membrane region, TM; *trans*-membrane region.
B. Primary sequence alignment of the catalytic domain of PstP and the human PP2C
Conserved residues are boxed. The amino acids of PP2C involved in the binding of the metal ions and the phosphate are indicated with a star. Secondary structural elements are indicated above the sequence.

suggesting a functional association with the STPK. Indeed, it has recently been described that the PrkC kinase and the PrpC phosphatase from *Bacillus subtilis* form such a couple *in vivo* with opposite effects on stationary-phase physiology (Gaidenko *et al.*, 2002).

The catalytic domain of PknB is a functional protein kinase

The full-length recombinant PknB protein has been previously characterized and shown to possess STPK activity (Avenue-Gay *et al.*, 1999). To allow detailed biochemical and structural studies, we have chosen to focus on its catalytic domain. Multiple sequence alignment with members of the Ser/Thr protein kinase family and homology modelling based on available three-dimensional structures pointed to Gly 279 as the last residue in the C-terminal α-helix of the catalytic domain. Thus, the domain corresponding to amino acid residues 1–279 of PknB ($PknB_{1-279}$) has been produced in *E. coli* as a soluble, monomeric His-tagged protein (Fig. 5A).

The kinase activity of $PknB_{1-279}$ was tested either in an autophosphorylation assay or using MBP as a model substrate. Like the full-length renatured PknB (Avenue-Gay *et al.*, 1999), $PknB_{1-279}$ autophosphorylates and phosphorylates MBP (Fig. 5A). Thrombin-digested $PknB_{1-279}$ (i.e. without the His-Tag) is also autophosphorylated, indicating that specific autophosphorylation sites exist on the PknB catalytic domain (data not shown). Kinase activity depends on divalent cations (Fig. 5A), $PknB_{1-279}$ showing a clear preference for Mn^{2+} versus Mg^{2+} ions (Fig. 5B). These observations demonstrate that, when separately expressed, the catalytic domain of PknB has intrinsic kinase activity, implying that other regions of the protein (such as the juxtamembrane region) are not required to stabilize an active conformation.

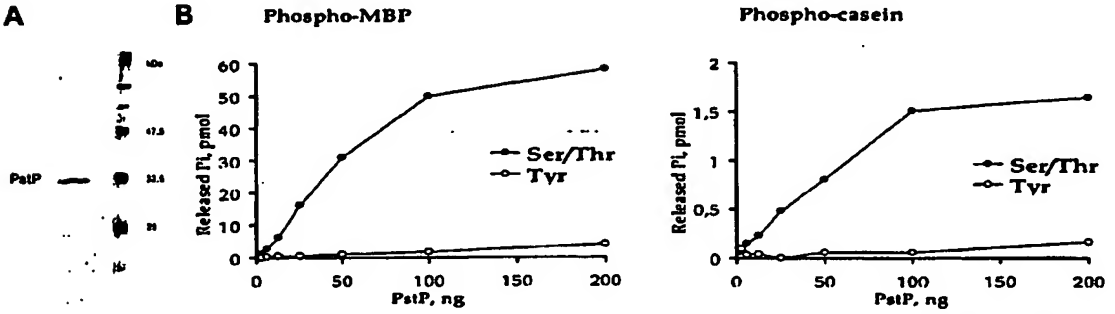

Fig. 3. A. Purification to homogeneity of $PstP_{1-240}$. His-tagged $Pstp_{1-240}$ was purified by affinity and size exclusion chromatography. The purity was then checked by SDS-PAGE electrophoresis. $PstP_{1-240}$ appears as a single discrete band on the gel with an apparent MW (32 kDa) slightly higher than the expected value (27.6 kDa).
B. Analysis of the specificity of $PstP_{1-240}$ towards phosphoresidues. MBP (left panel) and α-casein (right panel) were phosphorylated either on serine and threonine residues or on tyrosine residues with [γ-^{33}P]ATP. Release of radiolabelled inorganic phosphate was measured after incubation of increasing concentrations of the purified $PstP_{1-240}$ with the different phosphosubstrates.

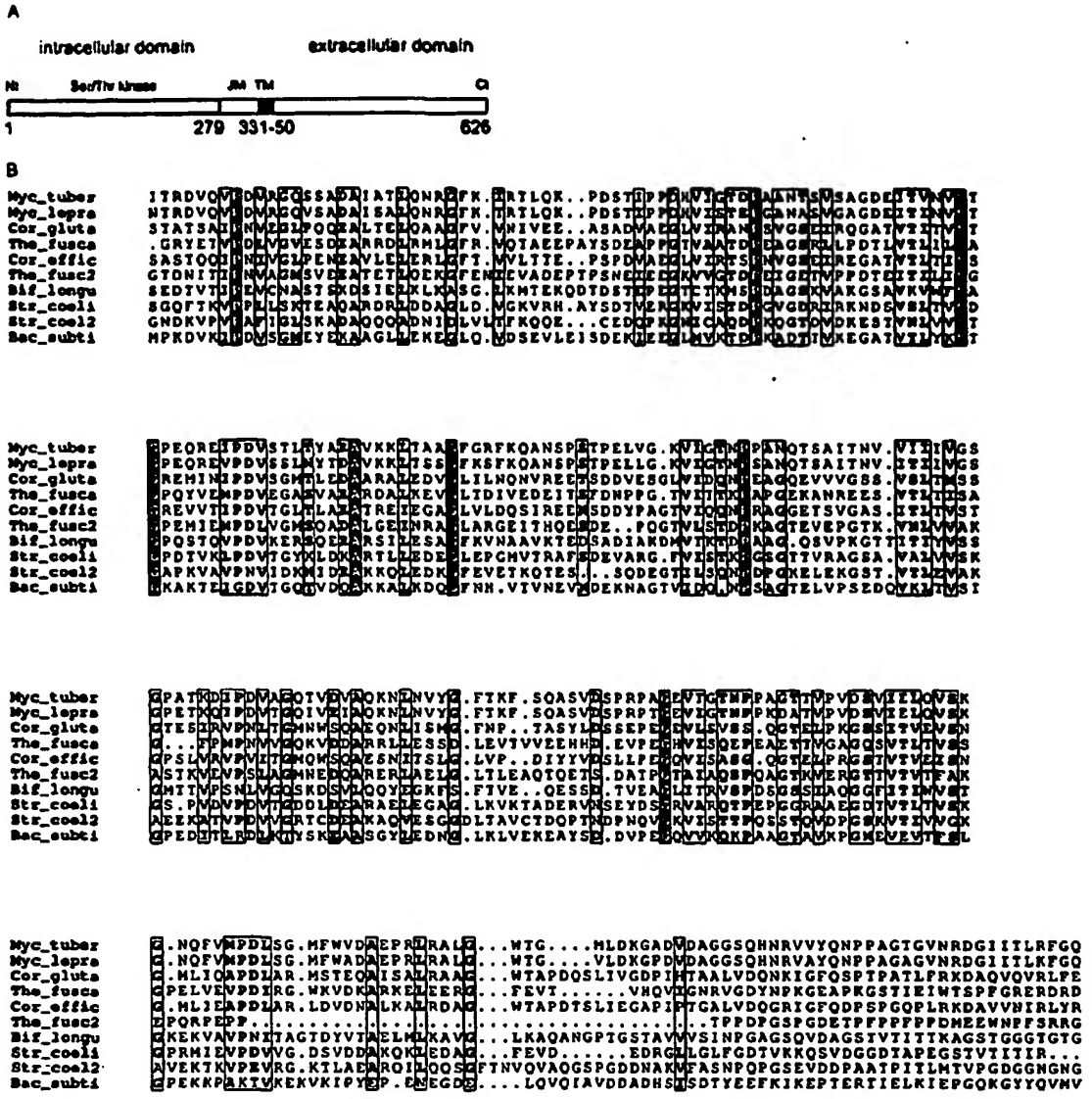

Fig. 4. A. Structural organization of PknB.
B. Sequence alignment of the putative sensor domain of bacterial STPKs. A BLAST search was conducted to detect the protein sequences most similar to the PknB C-terminal domain. We then selected among them the 10 STPKs most similar to *M. tuberculosis* PknB, i.e. STPKs from *M. leprae*, *Corynebacterium glutamicum*, *C. efficiens*, *Thermobifida fusca*, *Bifidobacterium longum*, *Streptomyces coelicolor* and *Bacillus subtilis*. The sequences of the C-terminal domains of these proteins were aligned with CLUSTALW. The extracellular domain of these STPKs consists of three to four PASTA domains, represented as different blocks. These repeated domains may have arisen by duplication events.

The recently determined structure of the catalytic core of PknB in complex with nucleotide at 2.2 Å resolution (Ortiz-Lombardía *et al.*, 2003) and 3 Å resolution (Young *et al.*, 2003) lends further support to these observations. The PknB catalytic domain was found to be very similar to its eukaryotic homologues and shares a number of essential hallmarks first described for PKA (Knighton *et al.*, 1991). In particular, all amino acid residues and

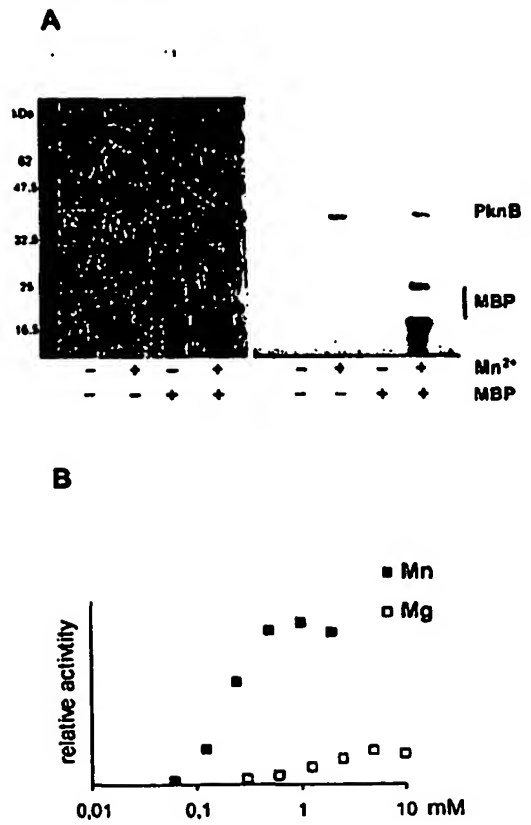

Fig. 5. A. Kinase activity of $PknB_{1-279}$: autophosphorylation and MBP phosphorylation assays. Purified $PknB_{1-279}$, alone or with the model kinase substrate MBP, was incubated with [γ-^{32}P]ATP in the presence or absence of $MnCl_2$. The reaction products were resolved on a SDS-PAGE gel that was Coomassie blue stained (left panel) then dried and autoradiographied (right panel). As observed for other phosphoproteins, the apparent MW of the protein in SDS-PAGE (40 kDa) is significantly higher than the expected value of 32 kDa.
B. Effect of divalent cations on the kinase activity of $PknB_{1-279}$. Various concentrations of $MnCl_2$ or $MgCl_2$ were used in the MBP phosphorylation assay. Relative quantification of the incorporated phosphate on MBP was obtained after Phosphorimager analysis.

other structural elements important for catalysis are found in their active conformation (Ortiz-Lombardía *et al.*, 2003).

Different preparations of $PknB_{1-279}$ produced a relatively broad complex mass peak in MALDI-TOF mass spectrometry experiments, with maximum intensity at m/z = 32 538 and smaller signals close to 80 Da, 98 Da or 160 Da apart (data not shown). After treatment with alkaline phosphatase, the peak shifted to m/z = 32 291 (the sequence-predicted average mass of uncleaved $PknB_{1-279}$ is 32 281 Da), indicating the presence of three phosphate groups linked to the protein (Fig. 6). However, we have failed to detect any phosphorylated residue in the 3D structure of PknB (Ortiz-Lombardía *et al.*, 2003). As the whole catalytic domain (except for residues A164-T179 covering most of the activation loop) is well-defined in the electron density map, this suggests that the putative phosphoresidues should be found in the disordered or mobile parts of the protein, i.e. at the N-terminal peptide extension outside the catalytic core and/or within the activation loop itself, in agreement with the putative phosphorylation sites recently proposed for this region by Young *et al.* (2003). [3]

PstP dephosphorylates PknB and inhibits its kinase activity

Full-length PknB has been shown to be autophosphorylated on Ser and Thr residues (Avenue-Gay *et al.*, 1999), and the question arises whether $PknB_{1-279}$ could be a substrate for PstP. To address this possibility, $PknB_{1-279}$ was autophosphorylated with radioactive ATP before incubation with PstP in the presence or absence of $MnCl_2$. As shown in Fig. 7A, PstP is capable of dephosphorylating PknB. Phosphate hydrolysis is also reflected by the shift in PknB migration on the gel concomitant with loss of

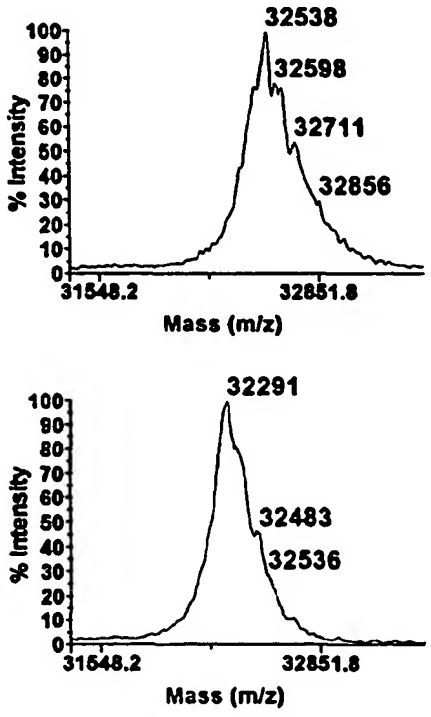

Fig. 6. MALDI spectra of PknB before (A) and after (B) PstP dephosphorylation.

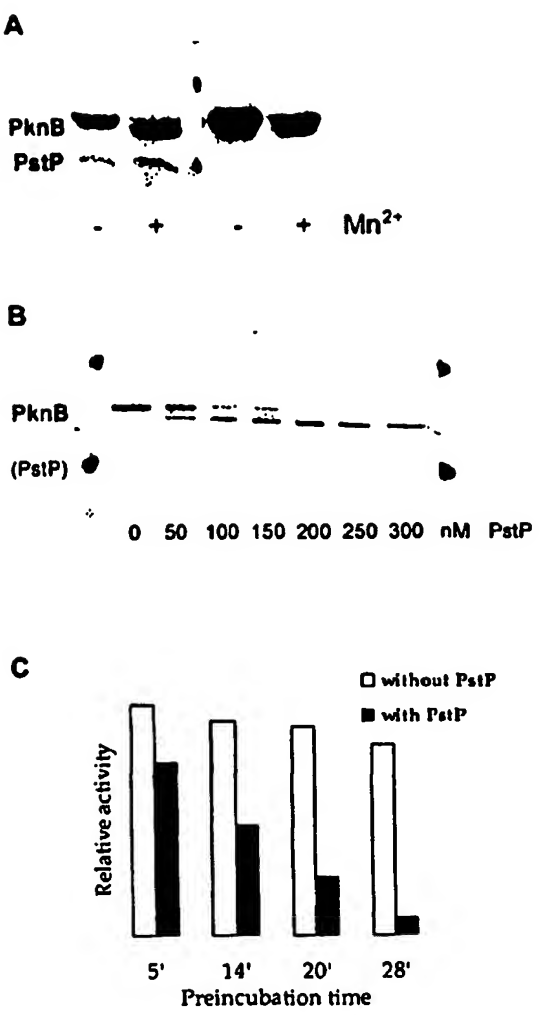

Fig. 7. Dephosphorylation assay using $PknB_{1-279}$ as a substrate for $PstP_{1-240}$ and effect of the dephosphorylation of $PknB_{1-279}$ by $PstP_{1-240}$ on its kinase activity.
A. Autophosphorylated $PknB_{1-279}$ in presence of [γ-^{33}P]ATP was used as substrate for $PstP_{1-240}$. As a control, $MnCl_2$ was omitted from the reaction buffer. The products of the reaction were subjected to electrophoresis on a denaturing gel. Left panel: the Coomassie blue stained gel; right panel: the autoradiograph.
B. Without prior labelling, dephosphorylation of PknB is followed with the shift in protein migration in SDS-PAGE.
C. $PknB_{1-279}$ was preincubated with $PstP_{1-240}$ for the indicated time. The kinase activity was then assayed using MBP and thio-γATP as substrates. Relative quantification of the kinase activity obtained with the PhosphorImager was plotted.

label, the lower band corresponding to dephosphorylated PknB. These differences in gel mobility were exploited to further monitor the phosphatase reaction without previous radioactive labelling (Fig. 7B). The dephosphorylation of PknB by PstP also indicates that the recombinant kinase produced in *E. coli* is phosphorylated *in vivo*.

We then asked whether the dephosphorylation of PknB could have an effect on its kinase activity. To address this question, PknB was preincubated with Pstp and ATP was replaced by thio-γATP in the kinase reaction. The rational for this assay resides in the ability of PknB of thiophosphorylating substrates whereas PstP is not active on these thiophosphosubstrates (data not shown). Under these conditions, the kinase activity can be measured without interference from the phosphatase activity. Figure 7C shows that prior dephosphorylation of PknB by PstP inhibits kinase activity on MBP. These results strongly suggest that the phosphorylation state of PknB is important in maintaining a fully active kinase.

Identification of two phosphothreonines in the activation loop of PknB

Mass spectrometry was used to identify the phosphoresidues detected in $PknB_{1-279}$. Comparison of the reverse-phase chromatograms of the trypsin digestion products of *either* $PknB_{1-279}$ or PstP-treated $PknB_{1-279}$ (covering 90% of the $PknB_{1-279}$ sequence) revealed changes in the elution pattern of some selected peptides (Fig. 8A). This observation was consistent with results from MS, in both reflector and linear modes, obtained from the corresponding whole peptide mixture (data not shown). In linear mode, two phosphopeptides could be identified from untreated $PknB_{1-279}$. A signal at m/z = 1850.1 was assigned to the His-tag peptide plus one phosphate group (calc. average mass = 1849.9 for the [MH]$^+$ peptide), and a strong signal at m/z = 2981.3 was assigned to the di-phosphorylated tryptic peptide A162-R189 (calc. mass = 2981.0), which includes a large fraction of the activation loop. It is noteworthy that no MS signal was detected for the non-phosphorylated A162-R189 peptide (calc. mass = 2821.1), except when $PknB_{1-279}$ was pretreated with a phosphatase such as alkaline phosphatase or PstP. Only in such conditions a prominent mass signal (at m/z = 2820.8) was observed in both linear and reflector modes.

These results were further confirmed when the separate peptide fractions were identified by MS measurements in reflector mode. Thus, peaks numbered 1 and 2 (Fig. 8A) were assigned to the monophosphorylated and unphosphorylated His-tag peptide, respectively, whereas peak 3 was assigned to the diphosphorylated A162-R189 peptide. Upon treatment with PstP, peak 1 was reduced in size, peak 2 increased and peak 3 almost disappeared, presumably giving rise to peak 4, which corresponds to the unphosphorylated A162-R189 peptide.

Post-source decay mass spectrometry (PSD-MS) measurement of a sample from peak 3 confirmed the presence of two phosphate groups in this peptide (Fig. 8B).

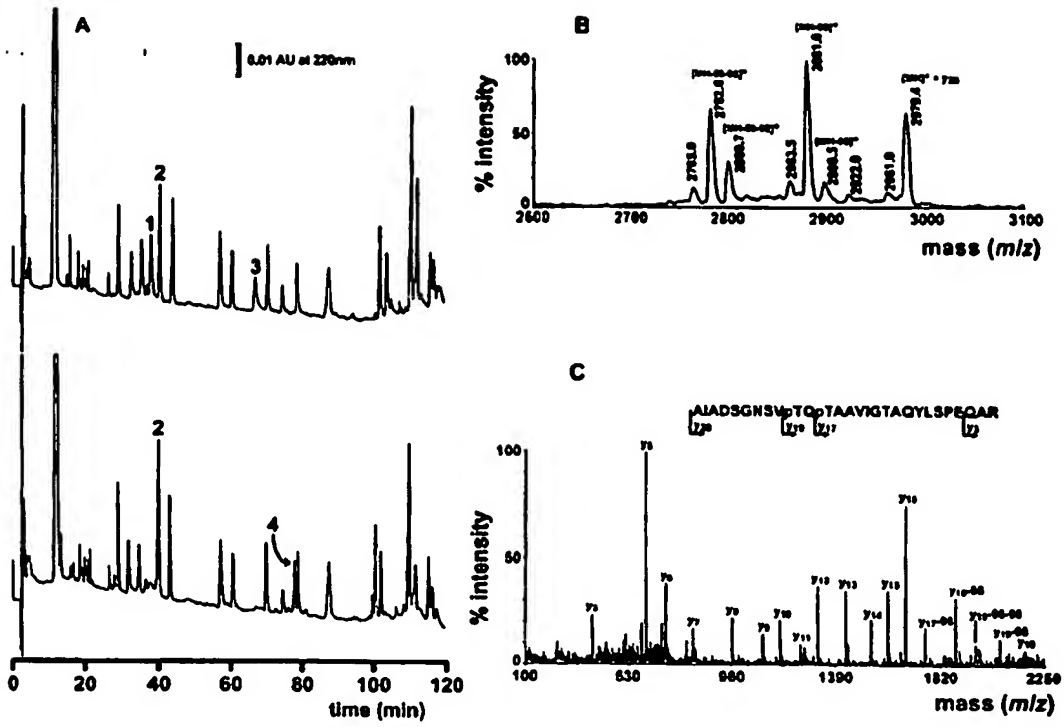

Fig. 8. Identification of phosphorylation sites in $PknB_{1-279}$.
A. HPLC separation of tryptic digests from $PknB_{1-279}$ before (upper panel) and after treatment with PstP (lower panel). Fractions were manually collected and analysed by MALDI-MS, with partial sequencing by PSD-MS when necessary for conclusive peptide identification. Only peptides relevant to this work are annotated in the chromatograms: peak 1, monophosphorylated His-tag peptide (*m/z* = 1848.61, calc. monoisotopic mass = 1848.84); peak 2, His-tag peptide (*m/z* = 1768.91, calc. monoisotopic mass = 1768.84, sequence GSSHHHHHHSSGLVPR); peak 3, diphosphorylated S162-R189 peptide (*m/z* = 2979.17, calc. monoisotopic mass = 2979.34); and peak 4, S162-189 peptide (*m/z* = 2819.5, calc. monoisotopic mass = 2819.41).
B. Detailed PSD spectra obtained with a sample from peak 3. The signals corresponding to −80 Da, −98 Da, -(80 + 98) Da, -(98 + 98) Da are strongly indicative of presence of two phosphate groups in serine and/or threonine residues in the analysed sample.
C. Integrated PSD spectra to confirm peptide identification by sequencing and to localise phosphorylated residues (measured values from the *y* -ion series in Da: y_5 = 374.0; y_6 = 600.1; y_8 = 687.2; y_7 = 799.8; y_8 = 962.0; y_9 = 1091.0; y_{10} = 1162.3; y_{11} = 1262.5; y_{12} = 1319.4; y_{13} = 1433.1; y_{14} = 1533.2; y_{15} = 1603.3; y_{16} = 1674.4; y_{17}–98 = 1757.3; y_{18}–98 = 1886.1; y_{18}–98–98 = 1969.0; y_{18}–98 = 2067.4; y_{19} = 2165.4).

Definitive identification and localization of the phosphorylated residues was achieved by PSD-MS sequencing of HPLC peak 3 purified from independent batches of PknB. This analysis showed that A162-R189 peptide was phosphorylated on Thr 171 and Thr 173 (Fig. 8C). In all cases, phosphorylation of these sites was close to 100%, indicating that these threonines are systematically and homogeneously linked to a phosphate. The HPLC patterns of PknB tryptic digests were extremely constant and reproducible over the time and with different preparations of the protein. However, in some experiments a shoulder or even a small peak could be observed, with a *m/z* = 3061.1 (data not shown). This was identified as a triphosphorylated species of the A162-R189 peptide (calc. mass = 3061.3). The third phosphosite is a serine that could not be unambiguously identified by sequencing and could correspond to either Ser 166 or Ser 169.

The above MS results identify two threonine residues from the activation loop, Thr 171 and Thr 173, as targets for PknB autophosphorylation and PstP dephosphorylation. These residues are part of a disordered region in the two PknB crystal structures (Ortiz-Lombardía *et al.*, 2003; Young *et al.*, 2003). However, inspection of the charge distribution at the molecular surface of the protein reveals an exposed cluster of basic residues that are favourably positioned to provide an anchoring site for the phosphothreonine residues (Fig. 9A). These arginine residues have partially disordered or mobile side-chains in the crystal structure, probably reflecting the absence of bound substrate. When compared with a similar cluster in PKA

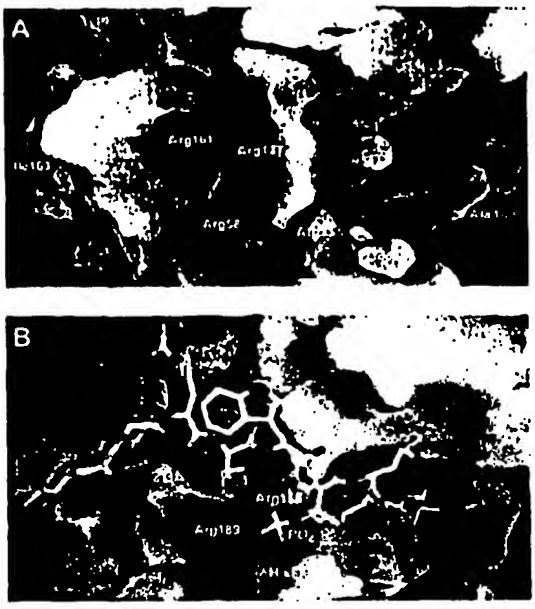

Fig. 9. The putative phosphate-binding site in PknB.
A. Surface representation of PknB (PDB code 1O6Y) colour-coded according to charge. A cluster of four exposed arginine residues could provide a binding site for the two phosphorylated threonine residues, Thr171 and Thr173. Sixteen residues from the activation loop (connecting Ile163 to Ala180 and including the two phosphothreonines) are disordered in the crystal structure.
B. Equivalent view of mouse PKA (PDB code 1ATP), in which the region corresponding to that missing in PknB is shown in stick representation. The phosphate group of phospho-Thr197 makes hydrogen-bonding interactions with the side chains of two arginine and one histidine residues.

(Knighton *et al.*, 1991) that binds phospho-Thr 197 in the activation loop (Fig. 9B), the positively charged region in PknB is found to cover a more extended surface area, raising the possibility of this region binding the phosphate groups of both Thr 171 and Thr 173.

Activation loop mutants of PknB

To confirm and further analyse the role of the identified phospho-threonines in PknB kinase activity, these residues were mutated to alanine, singly or in combination. The single mutants T171A, T173A and the double mutant T171/173 A were produced and analysed in the MBP phosphorylation assay. Comparison of the kinetics of phosphorylation of MBP by the mutants (Fig. 10) shows that the kinase activity is affected by each single mutation to a similar extent, being 15- and 20-times less active than PknB respectively. The double mutant is 300-fold less active, suggesting a combined effect of the two phosphothreonines on kinase activity. These results confirm that double phosphorylation of the activation loop is required for full kinase activity and demonstrate unambiguously the involvement of both phosphothreonines.

These mutants were also tested for the presence and localization of phosphorylated amino acid residues and the degree of phosphorylation at each site, following the same experimental protocol described above for the wild-type enzyme (Table 1). The N-terminal His-tag peptide showed a consistently lower degree of phosphorylation in the three mutants when compared to the wild-type enzyme, reflecting the lower activity of the mutants. As for the wild-type enzyme, the mutant T171A is mainly diphosphorylated in the activation loop, the residues involved being now Ser 169 and Thr 173. However, phosphorylation of Ser 169 does not restore wild-type activity and seems to play no functional role. On the other hand, the T173A mutant appears to be mainly monophosphorylated in Thr 171 (a much smaller HPLC signal could be assigned to a diphosphorylated species at residues Thr 171 and either Ser 166 or Ser 169). Analysis of peptides from the trypsin-digested double mutant T171/173 A demonstrated the occurrence of unphosphorylated (36%) and one monophosphorylated (at either Ser 166 or Ser 169) A162-R189 peptide species. In summary, both single mutants appear still fully phosphorylated on the remaining threonine and the activity decrease of the single and double mutants did not show co-operative behaviour, suggesting that Thr 171 and Thr 173 are independent phosphosites. Moreover, a similar decrease in kinase activity is observed upon the lost of each phosphosite, suggesting that the two phosphothreonines are equally important for PknB activity.

Discussion

Biochemical characterization of PstP and PknB

Although *M. tuberculosis* encodes 11 STPKs (Cole *et al.*, 1998) there is only one clear serine/threonine protein phosphatase, PstP which is a member of the PPM family (Bork *et al.*, 1996). We show here that its catalytic domain, $PstP_{1-240}$, dephosphorylates substrates previously phosphorylated on serine or threonine but not on tyrosine residues. Furthermore, its activity is strictly dependent on Mn^{2+} or Mg^{2+} ions, which is consistent with the deduced metal-ion catalysed dephosphorylation mechanism for this family (Das *et al.*, 1996).

On the basis of its amino acid sequence, PknB (and all other mycobacterial STPKs) have been classified in the Pkn2 family of prokaryotic STPKs (Leonard *et al.*, 1998), the cluster that most closely resembles their eukaryotic counterparts and that could have arisen by early horizontal transfer from eukarya to bacteria with complex development cycles. Recombinant full-length PknB has

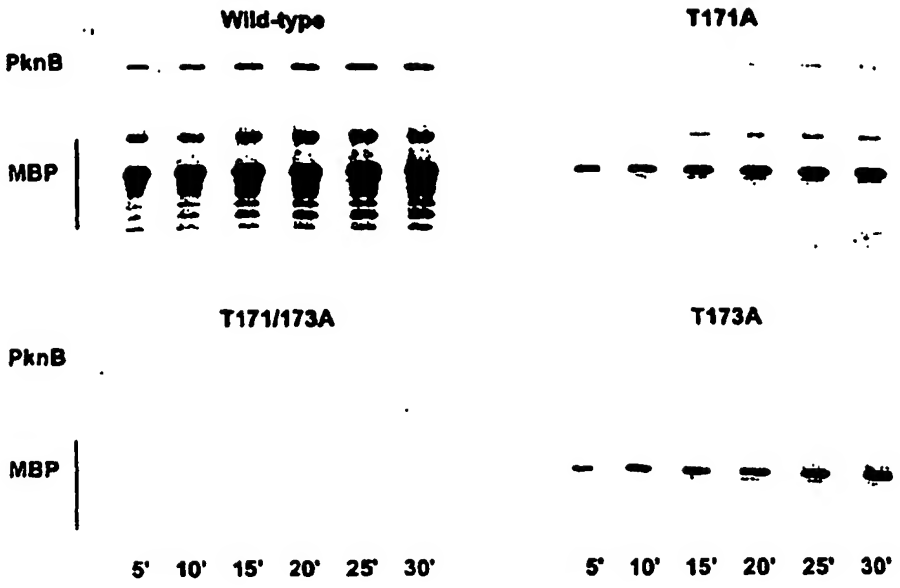

Fig. 10. Kinase activity of the activation loop mutants of PknB. MBP phosphorylation assays have been performed in parallel for the alanine mutants and the wild-type $PknB_{1-279}$. Relative quantification of the kinase activity was obtained with the PhosphorImager: T171A, T173A and T171/173 A mutants are ~15, 20, and 300 times less active than $PknB_{1-279}$ respectively.

already been shown to possess kinase activity and autophosphorylation sites on both serine and threonine residues (Avenue-Gay *et al.*, 1999). Here we studied a construct limited to the catalytic core domain, $PknB_{1-279}$, as defined by sequence homology. We found that this construct is an active kinase showing that the juxtamembrane region is not required for activity, although it may still be involved in further stabilization or activity regulation (see below).

PknB is regulated by phosphorylation of two Thr residues in the activation loop

Various mechanisms of eukaryotic protein kinase regula-

Table 1. Phosphorylation status of wild-type and mutants $PknB_{1-279}$.

	Phosphorylation status[a] and amino acid(s) involved[b]		
Protein	His-Tag peptide	Peptide S162-R189	Protein
PknB[c]	45–60% non-P	close to100% di-P	Thr171 and Thr173
	40–55% mono-P	trace of tri-P [d]	Thr171, Thr173 and (Ser169 or Ser166)
T171A	82% non-P	close to100% di-P	Thr173 and Ser169
	18% mono-P		
T173A	87% non-P	96% mono-P	Thr171
	13% mono-P	4% di-P	Thr171 and (Ser169 or Ser166)
T171/173 A	89% non-P	36% non-P	----
	11% mono-P	64% mono-P	(Ser169 or Ser166)

a. Refers to relative amounts of phosphorylated species present in Nt His-Tag peptide or in peptide S162-R189 populations. Non-P, mono-P, di-P or tri-P indicates absence, one, two or three phosphate groups present respectively. Peptide samples were isolated and quantified after protein treatment with trypsin followed by HPLC and peak identification by MS, as mainly described in Fig. 8 and in *Experimental procedures*.
b. Modified amino acid(s) by phosphorylation were localized in the sequence S162-R189 by PSD-MS as exemplified in Fig. 8B and C following the protocols described in *Experimental procedures*. The phosphorylated serine of the Nt His-Tag peptide (MGSSHHHHHHSSGLVPR) was not identified.
c. Samples from three independently produced batches of $PknB_{1-279}$ were tested.
d. The phosphorylation of the third residue in the activation loop, Ser 169 or Ser 166, appears of minor importance, as the degree of phosphorylation detected was systematically low or nul.

tion have been described (Johnson *et al.*, 1996; Hubbard and Till, 2000; Huse and Kuriyan, 2002). The transition between active and inactive forms may occur via control of access to the catalytic and/or the substrate-binding site, or by rearrangement of structural elements involved in catalysis or substrate recognition. Furthermore, interaction with other protein domains or cofactors may take place. It is noteworthy that a large number of these regulation mechanisms involve phosphorylation/dephosphorylation (inside or outside the catalytic domain) through an autocatalytic mechanism or by the action of other intervening kinases and phosphatases.

The present study shows that the catalytic domain of PknB autophosphorylates *in vitro* and is phosphorylated when expressed in *E. coli*. To see whether PknB autophosphorylation could play a regulatory role, we first identified phosphorylated residues in PknB. Mass spectrometry analysis indicated that two threonines residues of the activation loop (Thr 171 and Thr 173) are systematically phosphorylated (presumably autophosphorylated). Other eukaryotic protein kinases also display two phosphorylation sites in their activation loops, such as MKK1 (two Ser residues, Alessi *et al.*, 1994) or ERK2 (a Thr and a Tyr residues, both of which have to be phosphorylated to form the active enzyme, Robbins *et al.*, 1993). The activation loop is a major control element of an active/inactive conformational switch in numerous kinases (Steinberg *et al.*, 1993; Johnson *et al.*, 1996; Huse and Kuriyan, 2002) whose conformation often depends on their phosphorylation state (Johnson *et al.*, 1996). From its structural location, this loop may control both the accessibility to the catalytic site and the binding of the substrate. A broad range of regulatory properties has been assigned to this loop, such as contributing to the proper alignment of the catalytic residues, correcting the relative orientation of the two lobes, permitting substrate binding and/or stimulating ATP binding (Huse and Kuriyan, 2002).

The inhibitory effect of dephosphorylation of PknB on its kinase activity shows that phosphorylation is required for full activity. This is further confirmed by the mutagenesis study of activation loop threonine residues. Compared to the wild-type enzyme, the two single mutants, still phosphorylated on the remaining threonine, display comparable, reduced activities whereas the double-mutation further decreases the activity. Hence, Thr 171 and Thr 173 play independent and equivalent but complementary roles to reach maximal kinase activity.

The structural role of the phosphothreonine residues in PknB remains unexplained because the activation loop is disordered in the crystal structures (Ortiz-Lombardía *et al.*, 2003; Young *et al.*, 2003). This is not unusual in kinase structures. It has been observed both in active and inactive kinases, and does not indicate a particular phosphorylation state. In some kinases, phosphorylation of the loop fixes its conformation (Johnson *et al.*, 1996) and disorder could thus indicate partial phosphorylation. However, this does not seem to be the case for PknB as the activation loop has no defined structure in the crystal structure despite complete phosphorylation of both threonines. Instead, stabilization of the PknB loop could occur upon the binding of the peptide substrate through an induced-fit mechanism or by additional intra- or intermolecular interactions with other factors outside the kinase core. In any case, a positively charged region is observed in the PknB structure at the expected phosphothreonine-binding site, equivalent to a similar cluster that in PKA binds the single phosphorylated threonine, Thr197 (Fig. 9).

Taken together, these results strongly suggest that PknB kinase activity can be regulated by the state of phosphorylation of its activation loop *in vivo* through an autophosphorylation mechanism. Interesting observations can be drawn from the inspection of the activation loop sequences from the other *M. tuberculosis* STPKs. One or both threonines are conserved in all but two STPKs (PknG and PknI have shorter loops) suggesting that these enzymes should also be regulated by autophosphorylation in their activation loops. Thus, besides the same overall 3D structure and catalytic mechanism, eukaryotic and prokaryotic kinases would also share this mechanism of regulation, in spite of previous claims suggesting the absence of this process in prokaryotes (Motley and Lory, 1999). Further investigations are obviously required to determine the physiological relevance of PknB dephosphorylation by PstP and the effect of this protein phosphatase on other kinases, in particular PknA which is present in the same operon.

Other possible mechanisms of PknB regulation

Other mechanisms of kinase regulation could exist. PknB is presumed to be a transmembrane protein with a putative external ligand binding domain, an organization similar to that found in sensor histidine kinases (Parkinson, 1993) and receptor tyrosine kinases (Schlessinger, 2000). Binding of a ligand to the extracellular domain of the latter usually promotes receptor dimerization and/or a structural rearrangement that induces autophosphorylation and hence activation of the kinase domain. Interestingly, dimerization has recently been reported for PrkC (Madec *et al.*, 2002), a transmembrane STPK from *B. subtilis* with homology to PknB both in its Nt and Ct domains (Fig. 4B). Another regulation mechanism, described for both the type I TGF-β receptor serine/threonine kinase (Huse *et al.*, 1999) and the ephrin receptor tyrosine kinase (EphB2)(Wybenga-Groot *et al.*, 2001), involves the maintenance of an inactive state via the interaction of the

juxtamembrane region with the kinase domain. Upon ligand stimulation of EphB2, the autophosphorylation of Tyr residues in the juxtamembrane sequence releases the inhibition and renders this sequence available for further interaction with SH2 domains of target proteins (Wybenga-Groot *et al.*, 2001). The juxtamembrane region is missing in $PknB_{1-279}$. A recombinant construct of PknB corresponding to the catalytic core of the kinase plus the juxtamembrane sequence was also produced (see *Experimental procedures*). On preliminary analysis, three phosphorylation sites including Thr 294 and Thr 309 were identified in the juxtamembrane sequence (data not shown). Whereas the relevance of these phosphorylation events *in vivo* remains to be determined, it is worth noting that these phosphoresidues could also provide recruitment sites for specific Forkhead-associated (FHA) domains, see below.

PknB and PstP may regulate mycobacterial cell growth

In prokaryotes, genes involved in the same cellular process are frequently clustered often forming an operon. Thus, co-localization of the *pknB* and *pstP* genes in the same genomic region (Fig. 1) reinforces the hypothesis that these enzymes could intervene in the same signal transduction pathway. Furthermore, the organization of this genomic region suggests the participation of additional signal transduction elements, including a second STPK (namely PknA) and two proteins harbouring FHA domains (Rv0019c and Rv0020c), all of which are also conserved in other actinobacteria (Fig. 1). The FHA domains are small (Å 130 aa) protein modules that mediate protein–protein interaction via the recognition of a phosphorylated threonine on the target molecule (Durocher and Jackson, 2002). In eukaryotes, they are present in numerous signalling and regulatory proteins such as kinases, phosphatases, RNA-binding proteins and transcription factors. Rv0019c (155 aa) corresponds to a single FHA domain whereas Rv0020c (527 aa) has two domains, a Ct FHA domain and a Nt domain that shows no homology with any known protein except with its orthologue in *M. leprae* (ML0022). The FHA domain of Rv0020c has recently been characterized for its ability to bind phosphorylated peptide ligands (Durocher *et al.*, 2000).

Also found in the same conserved operon (Fig. 1) are two genes, *pbpA* and *rodA*, encoding proteins involved in controlling cell shape and peptidoglycan synthesis during cell growth (Matsuhashi, 1994). Cell growth and development require the cell wall to have a dynamic structure. Indeed, the cell wall changes continuously, during growth and developmental processes such as sporulation, and in response to changes in the environment. Moreover, morphological adaptation like cell wall thickening could be an important determinant for survival of the slow-growing pathogenic mycobacteria in anaerobiosis (Cunningham and Spreadbury, 1998). Cross-linked peptidoglycan, a major component of the bacterial cell wall, is synthesized by penicillin-binding proteins (PBP), which are membrane anchored enzymes with two external catalytic modules. Some PBPs are only involved in specific phases of growth or development and, for transglycosylase activity, they are each associated with a membrane protein partner. Thus in *E. coli*, PBP2 and RodA are responsible for peptidoglycan synthesis during cell elongation and for determination of the rod shape, whereas PBP3 and FtsW are involved in peptidoglycan synthesis during cell division (septation). In *B. subtilis*, a homologous couple (PBP and SpoVE) is thought to be engaged in spore formation.

One reasonable working hypothesis, that is currently being tested, involves PknA, PknB and PstP, along with other signalling modulators, co-ordinately regulating cell elongation during growth. Indeed, recent data suggest a regulatory role for PknA in cell elongation (Chaba *et al.*, 2002) and it has been speculated that the extracellular domain of PknB could bind unlinked peptidoglycan (Yeats *et al.*, 2002). Kinases and phosphatase might have opposing effects on the control of such a complex integrated pathway. Tight regulation of the process of cell elongation could therefore be a key element in mycobacterial development and provide a link between the intra/extracellular growth phase and the latent lifestyle within the granuloma. If this model is correct, inhibitors of STPK, or even PstP, would represent attractive lead compounds for development into antitubercular agents capable of targeting *M. tuberculosis* in the different stages of its life cycle.

Experimental procedures

Sequence analysis and modelling

For biochemical and structural (Ortiz-Lombardía *et al.*, 2003) studies, the catalytic kinase core of PknB was originally defined using a homology modelling approach. The 10 closest sequences from the Protein Data Bank were selected, and a multiple alignment was carried out using CLUSTALW. After manual editing of the alignment, the five sequences sharing highest identity with PknB (namely *C. elegans* Twitchin kinase, rabbit phosphorylase kinase, mouse PKA, and human CDK6 and CDK2) were used as templates for homology modelling. Using different combinations of these templates various families of models were constructed and refined with the program MODELLER (v. 4.0). A comparison of the most self-consistent models allowed us to identify Gly 279 as the likely end point for the α-helix I defining the C-terminus of the kinase catalytic core.

Cloning and mutagenesis

Cosmid MTCY10H4 containing *pknB* (Rv0014c) and *pstP* (Rv0018c) was used in subcloning experiments. A PknB con-

struct corresponding to the putative cytoplasmic domain (catalytic domain + juxtamembrane sequence – aa 1–331) was first obtained, as some regions outside the kinase core could stabilize the catalytic domain. The following primers were used for PCR amplification: forward primer (with *NdeI* site): 5'-GATAGCCATATGACCACCCCTTCC-3' and reverse primer (5'-TAA codon + *Hin*dIII site): 5'-AAACCGAAGCTTAACGGC CCACCG-3'. The digested and purified PCR product was ligated into the pET28 expression vector using the engineered *Nde*I and *Hin*dIII sites. $PknB_{1-331}$ was expressed as a broad heterogeneous protein, probably reflecting heterogeneity of its phosphorylation state as various phosphorylated residues were detected in the juxtamembrane region (data not shown). A shorter construct corresponding to the core catalytic domain (aa 1–279) was thus obtained, introducing a stop codon by site-directed mutagenesis. PknB mutants (T171A, T173A, T171/173 A) were all obtained from this last construct by the same method.

The complete *pstP* gene was subcloned into pET28 expression vector using the following primers: forward primer (with *NdeI* site): 5'-CGGGGGCATATGGC GCGCGTGA-3' and reverse primer (TAA codon + *Hin*dIII site): 5'-GCAGTCGTAAGCTTATGCCGCCG-3'. The construct corresponding to the catalytic domain of PstP (aa 1–240) was then obtained by introducing a stop codon through site-directed mutagenesis.

All mutagenesis was done according to the Quick Change Stratagene procedure. Enzymes were purchased as follows: the T4 DNA ligase, *Nde*I and *Dpn*I restriction enzymes from Biolabs, *Hin*dIII and *Bgl*II restriction enzymes from Pharmacia, the *Pfu* and *Plu* turbo polymerases from Stratagene. All constructs were verified by DNA sequencing.

Protein expression and purification

Escherichia coli BL21 (DE3) bacteria transformed with the appropriate plasmid were grown at 37°C until late log phase in Luria–Bertani (LB) medium with antibiotic (kanamycin 30 μg ml^{-1}). Induction of expression was conducted for 12–16 h at low temperature (15°C) after addition of 1 mM IPTG. Bacterial pellet was resuspended in 50 mM Hepes buffer pH 7, 0.2 M NaCl, in the presence of protease inhibitors and sonicated. The lysate was cleared by centrifugation (20 000 *g*, 30 min to 1 h). The supernatant containing soluble proteins was applied to Ni-column (Pharmacia) using an FPLC system and eluted by an imidazol gradient (0–0.5 M). A further step of gel filtration (Superdex 75) was required to separate the aggregated material from the monomeric proteins and to remove imidazol and most of the Ni^{2+} cations. Proteins were subsequently concentrated by means of Macro- and Micro-sep concentrators (Pall/Gellman). Protein concentration was determined using the Bio-Rad protein assay. Purity of the samples was checked by SDS-PAGE electrophoresis.

Protein kinase assays

The kinase assays were carried out in 20 μl of kinase buffer (Hepes 50 mM pH 7, DTT 1 mM, Brij35 0.01%) containing 2 mM $MnCl_2$, 100 μM ATP and 1 μCi of [γ-^{33}P]-ATP. For the analysis of divalent cation preference various concentrations of $MnCl_2$ or $MgCl_2$ were used, as indicated in the Fig. 1B. For autophosphorylation 5 μM final of the purified PknB was used. For phosphorylation of the MBP substrate by PknB or the PknB mutants, the enzyme/substrate ratio was 1:20 with 0.5 μM kinase. The reaction was started with the addition of the kinase and conducted at 30°C for 10 min. For the kinetics of MBP phosphorylation by PknB and the PknB mutants, 10 μl-aliquots of a scaled-up 60 μl reaction mixture were withdrawn at each indicated time. The reaction was stopped by the addition of SDS-PAGE sample buffer plus EDTA (25 mM final). Ten μl of the reaction were subjected to electrophoresis. In each case, the reaction products were separated on a 12% SDS-polyacrylamide gel and the radiolabelled proteins visualized by auto-radiography. To obtain relative quantification of the incorporation of radiolabelled ATP, the radioactive samples were also analysed using a Phosphorimager apparatus (STORM, Molecular Dynamics). For testing kinase activity of PknB after various incubation times with PstP, ATP and [γ-^{33}P]ATP were replaced by thio-γATP and [^{35}S]ATP-γS respectively. [γ-^{33}P]ATP and [^{35}S]ATP-γS were purchased from AmershamBiosciences. MBP was from Invitrogen.

Protein phosphatase assays

Dephosphorylation of phosphoSer/Thr or phosphoTyr proteins by PstP was assayed using either MBP or α-casein (SIGMA). Phosphorylated [^{33}P]Ser/Thr-substrates or [^{33}P]Tyr-substrates were prepared by phosphorylation of the proteins using either the catalytic subunit of PKA or the Abl protein tyrosine kinase. In each case, the kinase reaction was performed in 200 μl of buffer (50 mM Hepes pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij35) with 1 mM ATP, 75 μCi [γ-^{33}P]ATP, 200 μM substrate and 25 units of PKA or 10 units of Abl kinase. The reaction was incubated for 5 h at 30°C. Phosphorylated substrate was recovered by TCA precipitation and extensively dialysed at 4°C against a 25 mM Tris buffer pH 7.5 with 0.1 mM EDTA, 2 mM DTT and 0.01% Brij35. Dephosphorylation assays were carried out in a 25 μl reaction mixture containing 50 mM Hepes buffer pH 7.5, 0.1 mM EDTA, 1 mM DTT and 0.01% Brij35, 5 mM MnCl2. Phosphorylated [^{33}P] substrates were used to a final concentration corresponding to 10 μM of incorporated phosphates. The reaction was started with the addition of various concentrations of the purified PstP (up to 200 ng/25 μl, ≈ 0.3 μM) and incubated for 10 min at 30°C. The reaction was terminated by adding cold 20% TCA. After centrifugation, soluble materials were added to scintillation fluid and counted for the release of inorganic phosphate. The serine/threonine phosphatase PP1 and the Tyrosine phosphatase T-Cell PTP were used as control for the dephosphorylation of the phosphoSer/Thr substrates and the phosphoTyr substrates, respectively (not shown). The dephosphorylation of PknB by PstP was first performed using autophosphorylated [^{33}P]-PknB that was prepared according to the above protocol, except that no extra kinase was added. The reaction was performed in 15 μl of Hepes buffer 50 mM pH 7, DTT 1 mM, Brij35 0.01% with 2 mM $MnCl_2$. [^{33}P]-PknB and PstP were used at 5 μM and 1 μM, respectively, and incubated 30 min at 30°C. The reaction products were resolved on a SDS-PAGE gel and the lost

of labelling was visualized on the auto-radiography of the dried gel. The dephosphorylation of PknB by PstP was also simply assayed by the appearance of a lower band on a gel corresponding to dephosphorylated PknB. The reaction was carried out in 10 µl of the same buffer for 10 min at 30°C, except that PknB substrate was used at 1 µM, various concentrations of the phosphatase PstP were added from 50 to 300 nM.

Mass spectrometry analysis

Identification of phosphorylated sites was performed by mass measurements in whole peptide mixtures and in purified HPLC fractions of proteins digested with trypsin (Promega, 0.5 µg per 20–50 µg of protein sample in 50 mM ammonium bicarbonate buffer, pH 8.4, overnight incubation at 35°C). Twenty-six tryptic peptides covering 90% of the $PknB_{1-279}$ sequence were thus identified (data not shown), whereas digestion peptide products smaller than five amino acid residues could not be detected. In some experiments proteins were treated with a phosphatase before proteolytic cleavage: alkaline phosphatase from Roche Diagnostics (20 enzyme units per 20–40 µg of protein incubated in an assay mixture according to instructions supplied by the manufacturer, for 1 h at 35°C) or purified PstP enzyme as described elsewhere in this section.

MALDI-TOF MS was carried out in a Voyager DE-PRO system (Applied Biosystems) equipped with a N_2 laser source (λ = 337 nm). Mass spectra were acquired for positive ions in linear and reflector modes at an accelerating voltage of 20 kV. The matrix was prepared with α-cyano-4-cinnamic acid for peptides or with sinnapinic acid for proteins, as saturated solutions in 0.2% trifluoroacetic acid in acetonitrile-H_2O (50%, v/v). Measurement of peptide masses in reflector mode was performed under conditions of monoisotopic resolution with the accuracy close to 50 p.p.m. attained with external calibration. For this purpose a mixture of the following peptide mass standards was included ([MH]$^+$ monoisotopic mass, concentration): angiotensin I (1296.68, 2 pmol µl^{-1}); ACTH 1–17 clip (2093.08, 2 pmol µl^{-1}); ACTH 18–39 clip (2465.20, 1.5 pmol µl^{-1}); and ACTH 7–38 clip (3657.93, 3 pmol µl^{-1}). Better accuracy was obtained when internal mass calibration was sometimes performed with already characterised peptides present in PknB tryptic digests. For mass measurements of PknB proteins in linear mode, enolase of Baker's yeast (average mass of the protonated molecular ion $[MH]^+$ = 46.672, and $[MH2]^{+2}$ = 23.336) was used as a calibration standard. Samples for MS were usually prepared by spotting 0.5 µl of matrix solution and 0.5 µl of peptide solution, or tiny droplets from a desalting microcolumn eluted with matrix solution (see below), directly on the sample plate.

Selected peptides isolated from HPLC runs were sequenced by PSD-MS analysis of the *y* ion series generated from the samples (Kaufmann *et al.*, 1993), following instructions provided by the instrument manufacturer. When additional data were required to confirm a phosphorylation site by MS sequencing, the corresponding tryptic peptide was submitted to $Ba(OH)_2$ treatment for dephosphorylation of serine or threonine residues, following published procedures (Resing *et al.*, 1995).

HPLC separations were performed in a reverse-phase column (Vydac C18, 150 × 2.1 mm) equilibrated with 0.1% trifluoroacetic acid in H_2O (solvent A), and eluted with a gradient of 0.07% trifluoroacetic acid in acetonitrile (solvent B). Chromatographic conditions were as follows: flow rate 0.2 ml min^{-1}; chart paper 2 mm min^{-1}; gradient was from 0 min to 20 min up to 10% B, from 20 min to 100 min up to 30% B, from 100 min to 110 min up to 50% B, from 110 min to 115 min up to 100% B, and then 100% B isocratic for 5 min more; detection was by UV recording at 220 nm.

Relative amounts of the tryptic peptide A162-R189 showing different degrees and patterns of phosphorylation were calculated for wild-type and mutant PknB enzymes (Table 1). Peak size of purified and identified HPLC peaks (according to MS and PSD-MS measurements) was measured and corrected according to the chromatographic response of each peptide, tested in advance under identical chromatographic conditions as described above. The HPLC patterns of PknB tryptic digests were extremely constant and reproducible over the time and with different preparations of the protein. However, in some experiments a shoulder or even a small peak could be observed (data not shown). This was identified as the triphosphorylated species of the A162-R189 peptide. The third phosphosite is a serine that could not be unambiguously identified and could correspond to either Ser 166 or Ser 169.

For mass measurements, HPLC fractions were sometimes concentrated under a N_2 gas flow, freeze-dried, or immobilised on reverse-phase Poros 10 R2 beads (Applied Biosystems). The latter was also a useful procedure to desalt small peptide or protein samples in batch or in home-made microcolumns (Gobom *et al.*, 1999). Virtual tryptic digestions and other mass calculations were performed with the GPMAW32 (v.4.02) program (Lighthouse Data).

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Provisional CLAIMS

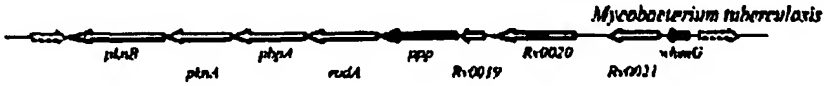
1) A bacterial operon comprising at least *PknB* and *PstP* genes

2) A bacterial operon according to claim 1, furthers comprising *pbpA* gene and *rodA* gene.

3) A bacterial operon according to claim 1 wherein the bacteria is a mycobacterium.

4) A bacterial operon according to claim 3, wherein the mycobacterium is Mycobacterium tuberculosis.

5) The mycobacterium operon corresponding to the genetic construction: (figure 1-manuscript)



6) A polynucleotide comprising an isolated *PstP* gene encoding for a PstP protein having a phosphatase activity or a variant thereof.

7) A purified PstP protein having a phosphatase activity or a variant thereof.

8) The purified PstP protein according to claim 7 having the sequence Rv0018c.

9) A polynucleotide comprising an isolated *PknB* gene encoding for a PknB protein or a fragment thereof having a substrate activity of the PstP protein according to Claim 8.

10) A polynucleotide according to claim 9 comprising nucleotide sequences encoding for the intracellular domain (corresponding to amino acid residues 1-279) or its catalytic domain (corresponding to amino acid residues 164-179) of the PknB protein.

11) A purified or isolated PknB protein having the substrate activity of the PstP phosphatase according to claim 7.

12) The purified or isolated PknB protein according to claim 11 comprising an amino acid sequence selected among amino acid sequences: (sequences done in Figure 4B) or variants thereof (wherein the catalytic domain - residues 164-179- and namely residues Trh171 and Thr173 remain invariant)

13) A method for identification of a compound which exhibits an antibacterial activity comprising contacting at least one protein encoded by a gene from the bacterial operon according to claim 1 with a compound to be tested and detecting modulating activity of said protein.

14) A method for identification of a compound which exhibits an antibacterial activity comprising contacting of at least one protein encoded by a gene from the bacterial operon according to claim 2 with a compound to be tested and detecting modulating activity of said protein.

15) A method for identification of a compound which exhibits PstP modulating activity, comprising contacting a PstP protein with a compound to be tested and detecting modulating activity of PstP.

16) A method according to claim 15, wherein the detection of modulating activity comprises detection of dephosphorylation activity of PstP.

17) A method for identification of a compound which exhibits PstP modulating activity according to claim 15, further comprising contacting a PknB protein with a compound to be tested and detecting modulating activity of PstP.

18) A method according to claim 14, wherein the protein is PknB protein encoded by *PknB* gene and the modulating activity comprises detection of kinase activity of PknB.

19) A method according to claim 13 further comprising contacting a PstP protein with a test compound in the presence of PknB protein or fragments thereof comprising its intracellular domain (residues 1-279) or its catalytic domain (residues 164-179)

20) A method according to claim 19, wherein the PknB protein and the fragments thereof have threonine residues in positions 171 and 173.

21) A method according to claim 15 wherein the PstP protein is expressed by a genetically modified cell.

22) A method according to claim 17, wherein both PstP protein and PknB protein are expressed by the same genetically modified cell.

23) A method for the preparation of a compound having an antibacterial activity comprising the steps:
   a) identification of a compound having an antibacterial activity by putting it in contact with at least a protein encoded by a gene of the bacterial operon according to claim 2 and detecting modulating activity of said protein
   b) synthesizing the identified compound by any known method.

24) A complex capable to inhibit the growth of a mycobacterium comprising at least the PstP protein and a chemical product reacting with the active site of the PstP.

25) A mycobacterium containing the complex according to claim 24.

2-1-2 Abstract

Sequence comparisons indicate that the genome of M. tuberculosis include genes coding for 11 eukaryotic-like putative protein kinases and three eukaryotic-like putative protein phosphatases, some of which might be important for cell signaling. Given the complex life cycle of M. tb and the relative small number of genes coding for two-component systems in the genome, it is likely that some of these eukaryotic-type enzymes could play an essential role in cell signaling and thus represent potential therapeutic targets.

In our laboratory, we have cloned and expressed many of these proteins. In particular, we have focused our attention on a conserved operon that includes one putative protein phosphatase (PstP) and two Ser/Thr protein kinases (PknA and PknB), as well as two proteins containing FHA domains (known to be involved in mediating protein-protein interactions in cell signaling). This work is reported in a manuscript signed by Boitel et al, which has been accepted for publication in Molecular Microbiology, and all relevant scientific information is described there in detail.

Briefly, we have cloned and characterized biochemically PstP. We demonstrated that PstP is indeed a Ser/Thr protein phosphatase, whose activity depends on Mn ions, and belongs to the PPM family of eukaryotic phosphatases. Genomic analysis suggests that it is the only such enzyme in the whole mycobacterial genome, thus making of this protein a potentially interesting target for drug design. We also demonstrated that PstP is capable of dephosphorylating the Ser/Thr protein kinase PknB and regulate the kinase activity in vitro. We had previously determined the crystal structure of PknB and showed that the global folding and the catalytic machinery of the kinase is quite similar to those of the eukaryotic Ser/Thr protein kinases (Ortiz-Lombardia et al, 2003, J. Biol. Chem., 278, 13094-13100). By mass spectrometry studies, we identified two Ser residues in the activation loop of PknB (Ser171 and Ser173) as the substrates of PstP. We mutated these two Serine residues into Alanine and showed that the double mutant has a significantly reduced catalytic activity, thus confirming their direct regulatory role and suggesting a general mechanism for kinase regulation in mycobacteria.

The conserved operon also includes two other genes (rodA and pbpA) encoding morphogenic proteins involved in peptidoglycan synthesis during cell growth, strongly suggesting that PstP could regulate essential functions possibly related to cell growth or latency of mycobacteria, and thus represent a potential target for drug design. However, at present no clear physiological role has been demonstrated for any of the eukaryotic-like protein kinases and phosphatases in M. tuberculosis.

Work in progress (unpublished)

Crystals of PstP have been obtained, and the structure determination is in progress, which will provide essential information for virtual and experimental inhibitor screenings to search for potential inhibitors.

The genetic invalidation (knockout) of the corresponding genes in the operon is in progress.

Transfection of the pstP gene into E. coli cells is toxic for the bacteria, producing DNA condensation and possibly inhibiting cell septation. An inactive mutant of PstP is now being tested in E. coli, to confirm that the phosphatase activity is responsible for the observed toxicity in E. coli.

A screening for putative PknB inhibitors (using commercially available compounds) is currently in progress. Once obtained, these compounds will be used to investigate whether the inhibition of PknB could affect cell growth in M. tuberculosis cell cultures.

To reconstitute the putative signaling pathway in mycobacteria, we are using PknB (and other kinases) to phosphorylate cell extracts from M. tuberculosis and identify the putative physiological targets of the kinase by 2D electrophoresis and mass spectrometry.

The other signaling elements of the operon (PknA and the two FHA-containing proteins) are currently being produced in E. coli for further biochemical characterization.

APPLICATION DATA SHEET

APPLICATION INFORMATION

Application Type::	PROVISIONAL
Subject Matter::	UTILITY
CD-ROM or CD-R?::	NONE
Title::	PKNB KINASE ACTIVITY IS REGULATED BY PHOSPHORYLATION IN TWO THR RESIDUES AND DEPHOSPHORYLATION BY PSTP, THE COGNATE PHOSPHO-SER/THR PHOSPHATASE, IN MYCOBACTERIUM TUBERCULOSIS
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